Ligand structure of the divinylsulfone-based T-gel

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Abstract

The requirements for divinylsulfone (DVS)-based gels to act as thiophilic adsorbents, binding immunoglobulins in a salt-dependent manner have been examined. No differences in protein binding were observed for a DVS-activated gel reacted with mercaptoethanol (the T-gel), or for the same gel treated at high pH to hydrolyse the active groups and/or allow the formation of cross-links within the matrix, indicating that an O atom may be substituted for the thioether without affecting the thiophilic interactions. Extending the time of the activation reaction between DVS and the matrix results in increased amounts of sulfone attached to the gel, but decreased levels of active vinyl groups. When coupled to mercaptoethanol, these adsorbents bound more IgG than gels activated for shorter periods. This provides a convenient method to prepare thiophilic adsorbents of high capacity while minimising the amount of DVS used.

The immobilised vinylsulfone must be linked to an electron donating atom for IgG to bind. When the vinyl was instead reduced with sodium borohydride, protein binding was decreased. No IgG bound to amine-coupled DVS-activated adsorbents, perhaps due to an overall positive charge on these gels at pH 7.4.

The binding of human IgG to the adsorbents is dependent on ligand density, with little protein binding to gels having less than 16 μmol sulfone per ml. The binding increased with the ligand density above this level, with more than 25 mg IgG binding per ml to an adsorbent having 114 μmol sulfone per ml. The lack of binding at low ligand densities would be expected if the IgG must interact with two or more sulfone ligands to be retained on the adsorbent. © 1997 Elsevier Science B.V.

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1. Introduction

Thiophilic interaction chromatography was discovered by chance when it was observed that a divinylsulfone-activated gel, coupled to mercaptoethanol (the T-gel) bound protein from human serum in a salt-dependent manner [1]. When other low molecular weight thiols were substituted for mercaptoethanol, there was a similar pattern of protein

binding, but when ethanolamine was coupled to the activated gel, or the active groups reacted with hydroxyls, no protein binding was observed. To emphasise the importance of the sulfone and thioether sulfur atoms, this interaction was termed 'thiophilic'.

Thiophilic chromatography differs from hydrophobic chromatography in that different proteins are bound to the gels; for example hydrophobic adsorbents predominantly bind albumin from serum, while thiophilic adsorbents bind immunoglobulins [1]. Thiophilic interactions are independent of tem-

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perature [2–4] while hydrophobic interactions decrease with decreasing temperature. Both types of interactions are salt-promoted, but NaCl tends to decrease thiophilic interactions while strengthening hydrophobic interactions [5], suggesting that in thiophilic interaction chromatography there is a greater requirement for the salt to be structure-forming.

The mechanism of protein binding has been postulated to be through the formation of an electron donor–acceptor complex between adjacent electron-rich and deficient regions situated within a hydrophobic cavity on the protein, and the electron-donating thioether and electron-accepting sulfone of the ligand [6].

Despite the terminology 'thiophilic', neither sulfur atom in the T-gel is essential for producing thiophilic-like adsorption. Similar IgG binding to ligands that lack one or both of the sulfur atoms of the T-gel has been demonstrated [7–9]. However, all of the ligands shown to behave as thiophilic ligands have both electron donating and accepting groups.

The divinylsulfone-based T-gel was further examined. Analogues were synthesised, containing higher numbers of sulfones and thioethers and were shown to act with even stronger T-gel characteristics [10]. More recent work has indicated that it is the sulfone of the DVS-based gels rather than the structure of the coupled ligand which is responsible for the thiophilic interactions [4]. This has important implications for the use of the DVS activation method for making affinity adsorbents. Protein binding through interactions with the sulfone group may be wrongly attributed to the coupled ligand, and batch-to-batch variation in the properties of the coupled gel may occur if the activation reaction is not carefully controlled. In particular, it would be useful to know the relationship between the amount of protein binding through thiophilic interactions to a DVS-based adsorbent and the degree of activation of the gel, since the degree of activation is easily controlled by varying the amount of reagent and time of the reaction [11]. We decided to examine further the thiophilic properties of the DVS-activated gel, and of DVS-based gels where the active vinyl has been coupled to S, O or N, or reduced with borohydride.

2. Experimental

Sepharose CL-4B was purchased from Pharmacia Australia (Sydney, Australia). Divinylsulfone (DVS) and mercaptoacetic acid were from Sigma Aldrich Pty Ltd (Sydney, Australia). CSL (Melbourne, Australia) kindly supplied us with a sample of human IgG. All other reagents were of analytical grade. Selected gels were analysed by the Campbell Microanalytical Laboratory, University of Otago, New Zealand, using the oxygen flask method [12] for sulfur determinations and a Carlo Erba EA1108 Analyser for nitrogen analysis.

A series of DVS-activated gels were synthesised according to [11] by reaction at 20°C, at pH 12 in an equal volume of 0.5 M Na-carbonate buffer, with the amount of DVS and for the length of time given in Table 1. Other gels were reacted for longer times as indicated in the legend to Fig. 1. The degree of activation, that is the number of active groups introduced, was determined for each gel by reaction with 1 M mercaptoacetic acid, followed by titration of the acidic groups [11].

The DVS-activated gels were coupled with mercaptoethanol by reacting the activated gel with an equal volume of 0.2 M 2-mercaptoethanol in 0.2 M Na-carbonate at pH 11 for 1 h. Under these conditions all active groups were reacted, as determined from the absence of mercaptoacetic acid reactive groups remaining after the coupling. For comparison, the original T-gel was synthesised exactly as described [1], labelled P in Table 1. The activated gels indicated in Table 1 were analysed for total sulfur.

Samples of the highly-activated gel (gel 12, Table 1) were treated with an equal volume of 0.5 M carbonate at pH 12 for 8, 16 or 24 h at 20°C. The number of active groups remaining were determined then blocked by reaction with 2-mercaptoethanol as described above.

Portions of the same highly-activated gel were partially reduced by treatment with limited amounts of sodium borohydride (10, 20, 50 or 100 mM final concentrations) in 0.2 M Na-carbonate at pH 10 for 16 h at 25°C. The remaining active groups were determined and coupled with 2-mercaptoethanol. Samples of DVS-activated gels 6, 7, 9 and 11 (Table 1) were reduced by treatment with 100 mM sodium

Table 1
The effect of DVS activation conditions on the binding of IgG to the mercaptoethanol coupled adsorbents

Gel number	Amount of DVS (μl/g wet weight Sepharose)	Reaction time (h)	Active groups (μmol/ml)	Sulfone (μmol/ml)	IgG binding (mg/ml adsorbent)
1	1.7	1	2.1	nd	0
2	4	1	3.9	nd	0
3	9	1	8.4	nd	0
4	15	1	12.7	nd	0
5	22.5	1	16.5	nd	0.2
6	22.5	2	19.5	nd	3.5
7	35	2.5	26.6	nd	10.9
8	42.5	1.5	28.1	48.8	2.9
9	45	4	29.4	75.5	17.3
10	80	3	37.1	nd	5.2
11	80	6	48.2	nd	24.7
12	100	8	50.9	114.1	26.3
P	50 pH 11	18	20.0	71.0	7.9

Activation of Sepharose CL-4B was performed at pH 12, except for the T-gel of Porath (P) which was synthesised according to the original method with activation at pH 11 [1]. The amount of DVS used, and the time of the activation reaction are shown. The number of active groups, and hence concentration of mercaptoethanol, were determined by reaction with mercaptoacetic acid according to Ref. [11]. The amount of sulfone was calculated from the sulfur content and the density of the activated gel (Table 3) (nd=not determined). The amount of IgG binding to and eluted from each adsorbent is given.

borohydride as described above. The amount of active vinyl remaining was determined.

Portions of DVS-activated gels 6, 7, 9 and 11 (Table 1) were coupled with ethanolamine at pH 8 and pH 11 and with diethanolamine at pH 10 by reaction with an equal volume of 1 M amine in 0.2 M phosphate (pH 8) or 0.2 M carbonate (pH 10 or 11) for 16 h at 37°C. The number of active groups remaining after coupling was determined by reaction with dithiothreitol and analysis of the coupled thiol concentration with 5,5'-dithiobis(2-nitrobenzoic acid) [13] according to [14]. In addition, the amount of amine coupled was determined by nitrogen analysis for each of the amines coupled to DVS-activated gel 9 (Table 2).

All protein binding experiments were performed at 20°C on gels packed in 2-ml columns (47 mm×7.4 mm I.D.), equilibrated with 0.5 M Na$_2$SO$_4$ in 20 mM K-phosphate pH 7.4. IgG was applied to the columns at a concentration of 10 mg/ml in the equilibration buffer, at 0.2 ml/min, until the A_{280} of the effluent was 90% of that of the applied sample. The columns were then washed with the equilibration buffer until the effluent absorbance was less than 5% of that of

the applied sample. The bound protein was eluted firstly with 20 mM K-phosphate pH 7.4, then with 30% propan-2-ol in 20 mM K-phosphate pH 7.4. The concentration of protein in the breakthrough, wash and elution fractions was determined from the A_{280} (E_{280} 1.38 mg^{-1} ml^{-1} [15]). For the majority of the adsorbents, the IgG binding capacity was determined only once.

3. Results and discussion

A series of DVS-activated gels were synthesised and their properties are listed in Table 1. The degree of activation corresponds to the amount of mercaptoethanol on the final coupled adsorbent, as hydrolysis of the active groups is unlikely to contribute greatly to the loss of active groups during the coupling reaction [11]. The sulfone content was determined for a number of the activated gels from the sulfur content and the density of the gel (Table 3) and is in most cases much higher than the concentration of active vinyl groups.

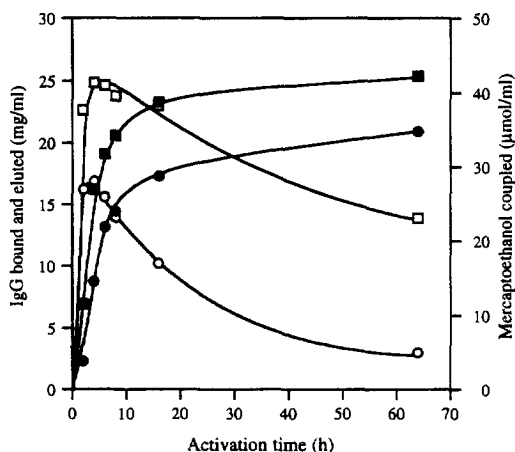The DVS reaction is complex, with side reactions

Fig. 1. Effect of DVS activation time on the amount of IgG bound to and eluted from the mercaptoethanol-coupled gels. Gels were activated with 40 µl or 80 µl of DVS per g wet weight of Sepharose for up to 64 h before being coupled with mercaptoethanol. The amount of coupled mercaptoethanol was determined from the number of active groups at each time and is given for both the 40 µl/g (○) and 80 µl/g (□) sets of gels. The IgG binding capacities are plotted for each gel in both the 40 µl/g (●) and 80 µl/g (■) series. The gel activated with 40 µl of DVS per g of gel for 16 h was found to contain 76 µmol sulfone per ml, while the gel activated with 80 µl DVS per g for 64 h was found to contain 181 µmol sulfone per ml (Table 3).

occurring simultaneously with the activation reaction. The sulfone remains attached to the matrix, but the second activated vinyl can be hydrolysed, or can react with a second matrix residue to form a cross-link [16], leading to a decrease in the number of active groups. It has even been suggested that the hydrolysed vinyl may react with a second DVS,

Table 2
Nitrogen analyses of the gel activated with DVS to 29.4 µmol/ml (Gel 9, Table 1) and coupled to ethanolamine at pH 8 or 11, or diethanolamine

Description of gel	Nitrogen content (w/w%)	Density of gel (mg/ml)	Total amine (µmol/ml)
Ethanolamine pH 8	0.77	40.4	22.2
Ethanolamine pH 11	1.12	39.9	31.9
Diethanolamine pH 10	0.95	41.1	27.9

The concentration of nitrogen in µmol per ml of gel can be calculated from the amount of N (w/w%) and the density of the coupled gel.

producing an ether-linked dimeric DVS structure [17], which may be involved in the binding of immunoglobulins [4].

The amount of IgG binding to and eluted from the mercaptoethanol coupled adsorbents is given in Table 1. The elution of bound IgG was greater than 95% for all of the coupled DVS-based gels used in this study. It can be seen that the amount of protein binding increases with the concentration of mercaptoethanol coupled to the adsorbent, where this is above about 16 µmol/ml, but below this amount of mercaptoethanol, no binding is observed. This is contrary to the dependence on ligand density observed for other pseudo-affinity adsorbents such as immobilised textile dyes [18–20] or immobilised metal affinity adsorbents [21,22] where protein binding is seen to increase with the ligand density at all low concentrations of ligand, but to plateau off, or even decrease at higher concentrations of ligand. Protein binding behaviour similar to that observed for IgG binding to the sulone-based T-gels has also been reported for immunoglobulins binding to the structurally unrelated thiophilic-like adsorbents consisting of 2-mercaptopyridine coupled to epichlorohydrin-activated supports [23]. This dependence on the ligand density could be explained if the immunoglobulin requires not one, but two ligands to enable binding to the thiophilic adsorbent.

The lack of correlation between protein binding and the degree of activation should be noted. It was observed that the gels binding higher amounts of protein for a particular degree of activation were those that were activated for longer times and thus had higher amounts of sulfone substituted onto the matrix. An adsorbent having 28.1 µmol active groups per ml was found to contain 48.8 µmol/ml sulfone and bind 3 mg IgG per ml of adsorbent, while one with 29.4 µmol/ml active groups had 75.5 µmol/ml sulfone and bound 17 mg IgG per ml, suggesting that the protein binding capacity is indeed highly dependent on the amount of sulfone on the gel. The sulfone that is in excess over the number of active groups must be coupled to an oxygen atom. According to Porath, this is not expected to contribute to protein binding [1,6].

To investigate the importance of the activation reaction time, two sets of DVS-activated gels were prepared, using 40 or 80 µl DVS per ml adsorbent,

Table 3
Sulfur analyses of a selection of the DVS-activated gels used in this study

Description of gel	Sulfur content (w/w%)	Density of gel (mg/ml)	Active groups (μmol/ml)	Total sulfone (μmol/ml)
Table 1, gel 8	3.88	40.4	28.1	48.9
Table 1, gel 9	6.16	39.3	29.4	75.5
Table 1, gel 12	8.13	45.0	50.9	114.1
Table 1, gel P	5.82	39.1	20.0	71.0
Fig. 1 40 μl/ml DVS, 16 h	6.72	36.1	17.0	75.6
Fig. 1 80 μl/ml DVS, 64 h	10.94	53.0	23.1	180.8
Fig. 3 100 mM NaBH$_4$	8.14	44.9	4.2	114.0

All determinations were made on activated rather than coupled gels, giving a measurement for the total sulfone introduced on DVS-activation. The concentration of sulfur in μmol per ml of gel can be calculated from the amount of S (w/w%) and the density of the activated gel, which increases with the degree of substitution [11].

and varying the activation time from 2–64 h. The number of active groups introduced was determined for each time point and the gels were then coupled to completion with mercaptoethanol. The protein-binding capacity of the coupled gels, together with the concentration of coupled mercaptoethanol is plotted against the activation time in Fig. 1. As expected [11] for both sets of gels, the degree of activation increased with time until it reached a maximum
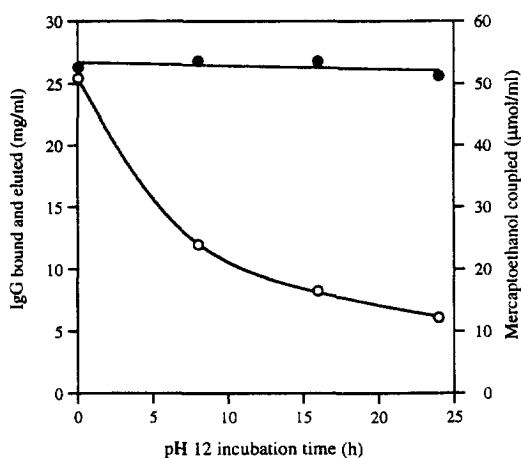

Fig. 2. Alkaline treatment of a DVS-activated gel and the effect on IgG binding to the coupled adsorbent. The highly-activated gel 12 (Table 1) was incubated at pH 12 in 0.5 M Na-carbonate for up to 24 h before being treated with mercaptoethanol. The number of active groups remaining after the incubation was determined and corresponds to the amount of mercaptoethanol coupled (○). The amount of IgG binding to each gel is shown (●).

value at about 4 h and then decreased due to cross-linking and hydrolysis. However, the IgG binding capacity of the mercaptoethanol-coupled adsorbents increased with reaction time for both sets of gels, with the final gels binding the maximum amounts of 25 and 31 mg IgG per ml adsorbent. These final gels, while containing less coupled mercaptoethanol than the earlier gels in each series, would contain more sulfone, either as cross-links or with the second vinyl hydrolysed [16]. The protein binding capacities follow the probable concentration of sulfone, suggesting that it is indeed the sulfone group that is primarily responsible for protein binding. This indicates in turn that O-linked vinylsulfones must also be able to contribute to protein binding.

In order to confirm that the thioether of mercaptoethanol is not essential for protein binding, a portion of the highly-activated gel 12 (Table 1) was treated with carbonate buffer at pH 12, for up to 24 h. This is the same buffer that is used for activating gels with DVS and so the same cross-linking or hydrolysis reactions would be expected to take place. For each time point, the number of active groups remaining was assayed and corresponds to the number of mercaptoethanol groups subsequently coupled to the gels. The capacity of these coupled gels for IgG was determined and together with the number of active groups, plotted against time, in Fig. 2. It is clear from this plot that the protein binding capacity of these gels is not dependent on the amount of mercaptoethanol coupled, since the capacity was

unchanged while the amount of mercaptoethanol coupled decreased from 50 μmol/ml to 12 μmol/ml. From this we conclude that an ether is as effective as a thioether in acting as an electron-donor in a 'thiophilic' complex.

In all the sulfone-based thiophilic adsorbents described, the immobilised active vinyl has been reacted with an atom that is able to act as an electron-donor. In order to determine whether this electron rich atom is necessary for IgG to bind to the adsorbent, samples of the highly-activated gel 12 (Table 1) were treated overnight with sodium borohydride (0–100 mM) to reduce the active vinyls to ethyl groups. Borohydride is a mild reducing agent, and unlikely to reduce sulfones to thioethers under these conditions [24]. The sulfur content was determined for both the initial activated gel, and the gel reduced with 100 mM borohydride, and found to be 114 μmol/ml in both cases, presumably as sulfone. The number of active groups remaining on the reduced gels was determined and corresponds to the amount of mercaptoethanol on the coupled adsorbent.

The amount of immunoglobulin G binding to the coupled gels was determined and plotted in Fig. 3, together with the amount of coupled mercaptoethanol, against the concentration of borohydride used to reduce the activated gel. The protein binding capacity decreased with the amount of mercaptoethanol coupled, and when the capacity is plotted against the mercaptoethanol concentration, the relationship is seen to be linear (Fig. 3, inset), with 27 mg of IgG binding to the adsorbent with 51 μmol mercaptoethanol per ml, extrapolating to 13 mg in the absence of coupled mercaptoethanol.

An electron-rich atom coupled to the vinylsulfone is clearly important for these gels to act as thiophilic adsorbents. It is possible that the ethylsulfone is able to interact with immunoglobulins to a small extent, but the residual protein binding not due to coupled mercaptoethanol is more likely to be due to the sulfone groups that have been hydrolysed or formed cross-links within the matrix. The original gel had 114 μmol/ml sulfone, but only 51 μmol/ml active groups, so all of the gels must have had at least 63 μmol/ml sulfone coupled to an oxygen atom.

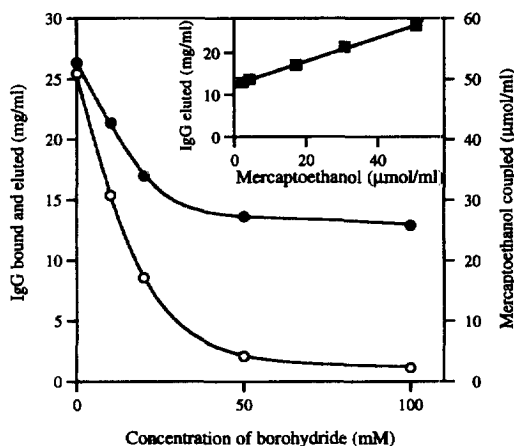Having observed a decrease in protein binding in the absence of an electron-donating atom coupled to

Fig. 3. Effect on protein binding of partial reduction of the active vinyl before coupling with mercaptoethanol. The highly activated gel 12 (Table 1) was partially reduced by treatment with sodium borohydride overnight at pH 10. The amount of coupled mercaptoethanol (○) was determined from the number of active groups remaining and is given together with the IgG capacity of the mercaptoethanol coupled gels (●) for each concentration of borohydride. The sulfone content of both the initial activated gel and of the gel treated with 100 mM borohydride was determined to be 114 μmol/ml (Table 3). Inset: The IgG capacity is plotted against the amount of mercaptoethanol coupled to the gels.

the immobilised vinylsulfone, we decided to investigate the ability of uncoupled DVS-activated gels to behave as thiophilic adsorbents. It has been reported that there is no significant difference in the immunoglobulin-binding behaviour of DVS-activated gels, or of these gels coupled with mercaptoethanol, thiosulfate or tryptophan [4]. A series of DVS-activated gels (Table 1, gels 6, 7, 9 and 11) were examined, but under the chromatographic conditions of 0.5 M Na$_2$SO$_4$ at pH 7.4, IgG bound to the gels, presumably covalently since it could not be eluted, masking any thiophilic interactions. These conditions provide a particularly effective and gentle way for immobilising antibodies, with as much as 50 mg IgG bound to the gel activated to 48 μmol/ml.

In order to examine amines as potential electron-donor atoms in thiophilic adsorption, portions of the same series of DVS-activated gels were coupled to ethanolamine at pH 8 or 11 and to diethanolamine. The coupling went to completion in all cases as determined from the absence of thiol-reactive groups at the end of the coupling reaction (not shown). The amount of amine coupled to the gel activated to 29.4

μmol/ml was determined in each case by nitrogen analysis (Table 2). The low level of ethanolamine coupled at pH 8, together with the absence of active groups remaining, under conditions where hydrolysis is negligible [11] suggests that approximately half of the ethanolamine is coupled to two DVS active groups, not one. That this is not observed when coupling occurs at high pH may be due to the relative concentrations of deprotonated primary amine in solution and deprotonated secondary amine coupled to the adsorbent. At the higher pH, all amines are predominantly deprotonated, and with 1 M amine, reaction proceeds smoothly with one amine coupling to each activated vinyl group. At the lower pH, much of the amine in solution is protonated (the pK_a for ethanolamine is 9.4) while the amine already coupled to the gel would be predominantly deprotonated (the pK_a of ethanolamine coupled to a DVS-activated gel is approximately 6 as determined by titration with HCl). In this case, the reaction of an already coupled ethanolamine to a second vinylsulfone would be favourable, if spatially possible.

The ability of these amine-linked DVS-activated gels to act as thiophilic adsorbents was compared with that of the same DVS-gels when coupled to mercaptoethanol, or with the vinyl group reduced with borohydride (Fig. 4). As expected, the thioether-linked adsorbents had the highest capacity for IgG, more than twice that of the borohydride treated gels, where the interaction is presumably due to the remaining O-linked sulfones. Surprisingly, the amine-linked adsorbents all bound even less protein than the corresponding reduced gels. This suggests that the amine coupled gel is not able to act as a thiophilic adsorbent, and may actually prevent binding to nearby O-linked sulfones, perhaps due to the presence of a net positive charge on the amine at pH 7.4. This would explain the differences observed for protein binding to the three amine-coupled adsorbents. The diethanolamine gel, and the ethanolamine gel coupled at pH 11 bind almost no protein, while the ethanolamine gel coupled at pH 8 is able to bind up to 5 mg IgG per ml. This final gel has less amine coupled, and much of this must be attached to two DVS groups, which would have the effect of further lowering the pK_a of the amine, so that there would be less positive charge associated with this gel than
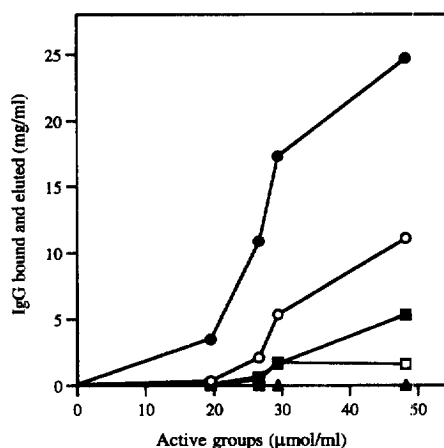

Fig. 4. Effect of ligand structure on protein binding to a series of DVS-based gels. The amount of IgG binding to the adsorbents is plotted against the number of active groups initially present for DVS-activated gels 6, 7, 9 and 11 (Table 1) coupled with mercaptoethanol (●), ethanolamine at pH 8 (■), ethanolamine at pH 11 (▲) and diethanolamine (□). The same DVS-activated gels were also reduced with 100 mM borohydride overnight. Less than 10% of the active groups remained after this treatment and the gels were not further reacted with mercaptoethanol. The amount of human IgG eluted from the reduced gels is given (○).

with the other two. That amine gels are such poor thiophilic adsorbents contradicts the original claims that these would be more effective than the O-linked gels [1,6].

4. Conclusion

The original T-gel introduced by Porath [1] consists of a DVS-activated gel coupled to mercaptoethanol and it was thought both S atoms were necessary for protein binding. In fact, no difference in IgG binding is observed for O- or S-coupled sulfone adsorbents: both are effective as thiophilic adsorbents. This can be exploited by greatly extending the DVS-activation time when synthesising a thiophilic adsorbent. The number of active groups and hence concentration of coupled mercaptoethanol decreases with time, but the amount of O-linked sulfone increases, producing a high capacity adsorbent while minimising the amount of reagent required.

In agreement with the original work, an electron-

donating species needs to be coupled to the vinyl-sulfone for immunoglobulins to bind. When the activated vinylsulfone is reduced to an ethylsulfone, protein binding is decreased, with the remaining protein binding presumably due to O-linked sulfones on the gel. This provides a useful route to blocking remaining active groups when the DVS activation method is used to immobilise ligands and thiophilic interactions are to be suppressed.

When amines are coupled to the DVS-activated support, IgG binding is decreased below the level observed for reduced gels. This may be due to electrostatic repulsion of the protein by an overall positive charge on the amines at pH 7.4. Where ethanolamine is coupled at lower pH, much of the amine appears to be coupled to two active groups. This tertiary amine would carry less positive charge at the pH of the chromatography; for this gel slightly more IgG is able to bind.

The concentration of S- or O-linked sulfone is very important for these thiophilic adsorbents. No binding of immunoglobulin G is observed for lower densities of sulfone, but at higher levels, binding increases rapidly. This can be explained if the T-gel requires not one, but two sulfone-containing ligands to interact with the IgG before it is retained on the adsorbent. The IgG-binding structure in the T-gel therefore requires two sulfone groups, in close proximity, coupled to either S or O, but not to N.

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